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PHOSPHOFRUCTOKINASE OF CULTURED AND AGED CARROT-ROOT SLICES

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Key Word Index—*Daucus carota*; Umbelliferae; *Helianthus tuberosus*; Compositae; tissue culture; ageing; phosphofructokinase; regulation of glycolysis.

Abstract—The properties of phosphofructokinase (PFK) of cultured and 'aged' carrot-root phloem and Jerusalem artichoke slices were studied. PFK activity was inhibited by ATP, citrate and phosphoenolpyruvate, and the plots of activity vs. fructose-6-phosphate concentration gave a sigmoidal curve. Sensitivity of PFK to ATP was not changed by ageing.

INTRODUCTION

MUCH information has been obtained about the allosteric properties of phosphofructokinase (PFK) [ATP: D-fructose-6-phosphate 1-phosphotransferase, E.C. 2.7.1.11], and it has been suggested that these properties may contribute to the regulation of glycolysis.[1–4] The change in properties of PFK has been reported by several authors.[5–8] Black and Wedding reported that the PFK from Jerusalem artichoke tuber discs 'aged' by washing was not inhibited by ATP and they suggested that respiratory rise during ageing might be due to the change in the sensitivity of PFK to ATP.[8]

In our previous studies on the respiratory metabolism during callus formation of the carrot root phloem slices cultured *in vitro*, it was found that respiratory rate increased remarkably at an early stage of culture.[9] In this paper, the properties of PFK preparations from the carrot root slices at various stages of culture or from aged ones were studied in order to know if an increase in respiratory activity might be caused by a change in the properties of PFK.

RESULTS

Changes in Respiratory Rate During Culture of Carrot-Root Slices

The rate of O_2 uptake in air[9] and the rate of CO_2 output in N_2 increased markedly during the first 4 days of culture, attained maxima between the 4th and 6th day (*ca.* 800 μl/g fr. wt/hr), and then decreased gradually (*ca.* 200 μl/g fr. wt/hr after 20 days).

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[8] M. K. BLACK and R. T. WEDDING, *Plant Physiol.* **43,** 2066 (1968).
[9] A. KOMAMINE, Y. MOROHASHI and M. SHIMOKORIYAMA, *Plant & Cell Physiol.* **10,** 411 (1969).

Properties of PFK Isolated from Slices at Various Stages of Culture

Some enzymic properties of PFK preparations from 0- (fresh slices), 4- and 14-day cultures were examined. The effects of ATP concentration on the activities of PFK from 0-, 4- and 14-day culture are shown in Fig. 1. These curves were of the same pattern, the optimal ATP concentration being 2·0 mM. The enzyme activity was reduced to about 50% of the optimum by 4 mM ATP.

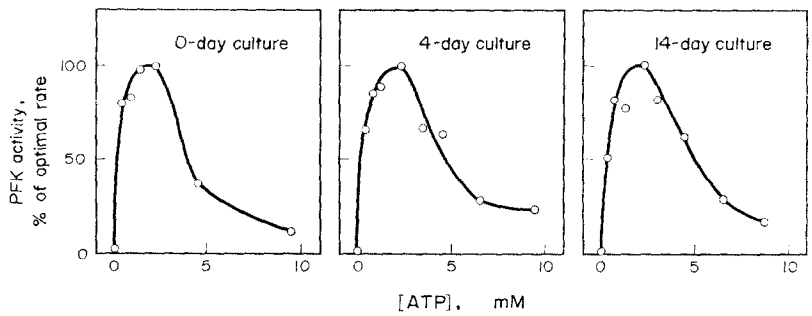

FIG. 1. EFFECT OF ATP CONCENTRATION ON THE ACTIVITY OF PFK FROM CARROT-ROOT PHLOEM SLICES AT VARIOUS STAGES OF CULTURE.

The effect of fructose-6-phosphate (F6P) concentration on the activity of PFK from the 4-day cultures was investigated in the presence of 6·6 mM ATP at pH 7·0. The plots of the initial reaction velocity vs. F6P concentration gave a sigmoidal curve, like that found by Dennis and Coultate.[10] Similar results were obtained with the PFK preparation from the slices cultured for 0 and 14 days. Citrate inhibited the activity of PFK preparations isolated from slices of 0-, 4- and 14-day-old cultures. Complete inhibition was caused by 20 mM citrate in the presence of 1·0 mM F6P and 1·0 mM ATP; 50% inhibition was found at 10 mM citrate. Pyruvate and malate had little effect at 20 mM. Phosphoenolpyruvate (PEP) also inhibited the activity of PFK preparation from slices at these stages of culture. PEP at 1·0 mM almost completely inhibited the activity. In 0-day culture 50% inhibition was found with 0·5 mM PEP and for 4- and 14-day cultures 0·25 mM caused 50% inhibition.

The optimum pH for PFK was found to be 8·0. The apparent K_m for F6P at the optimum pH was about 0·4 mM with enzyme preparations from slices at various stages of culture. The value was of the same order as those reported for the PFK from other plants under about the same conditions.[7,10]

Properties of PFK Isolated from Fresh and Aged Slices of Carrot-Root Phloem and Jerusalem Artichoke Tuber

The effect of ATP concentration on the activity of PFK from fresh carrot-root phloem slices (Fig. 1) and 20 hr aged slices, in which respiratory rate was about 2 times that of fresh slices, was almost the same. Respiratory rate of slices of Jerusalem artichoke tuber gradually increased with ageing until a peak (about 2 times the level of fresh slices) was attained at the 8–10th hr. Later it decreased again. The effect of ATP concentration on the activity of PFK from fresh slices and from 7 and 18 hr aged slices was found to be almost the same (Fig. 2). The effect of citrate on the enzyme from 7 hr aged slices is shown in Fig. 3(a). The

[10] D. T. DENNIS and T. P. COULTATE, *Biochem. Biophys. Res. Commun.* **25**, 187 (1966).

plots of activity vs. F6P concentration in the presence of 4 mM ATP at pH 7·0 gave a sigmoidal curve [Fig. 3(b)].

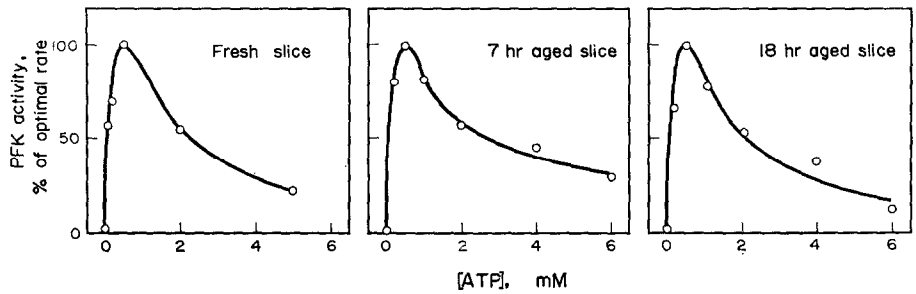

FIG. 2. EFFECT OF ATP CONCENTRATION ON THE ACTIVITY OF PFK FROM FRESH AND AGED SLICES OF JERUSALEM ARTICHOKE TUBERS.

DISCUSSION

Many authors reported that glycolysis can be regulated by allosteric effects of respiratory metabolites on the activity of PFK.[1–4] In higher plants, the activity of the enzyme is inhibited by ATP, citrate and PEP and the inhibition by ATP and citrate can be relieved by inorganic phosphate and F6P, but not by AMP, ADP and cyclic 3′,5′-AMP.[7,8,10–13]

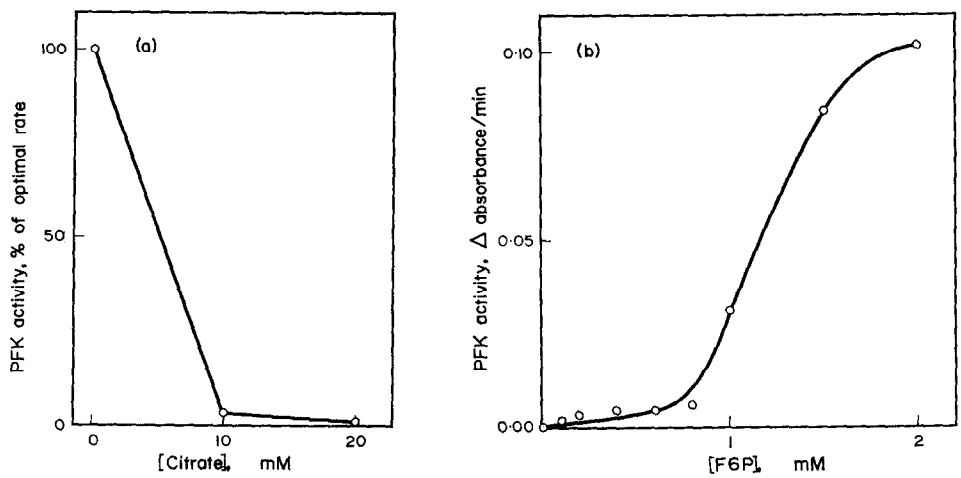

FIG. 3. EFFECT OF CITRATE ON THE ACTIVITY OF PFK FROM 7 HR AGED SLICES OF JERUSALEM ARTICHOKE TUBERS (a). EFFECT OF F6P CONCENTRATION ON THE ACTIVITY OF PFK FROM 7 hr AGED SLICES OF JERUSALEM ARTICHOKE TUBERS IN THE PRESENCE OF 4 mM ATP (b).

On the other hand, two forms of PFK, insensitive and sensitive to inhibition by ATP, were reported in yeast.[5] An ATP-sensitive form is desensitized on incubation with ADP, F6P, NH_4^+, Mg^{2+} and F^-.[6] In Brussels sprouts, Dennis and Coultate found that PFK

[11] O. H. LOWRY and J. V. PASSONNEAU, *Naunyn-Schmiedebergs Arch. Exp. Pathol. Pharmakol.* **248,** 185 (1964).
[12] G. J. KELLY and J. F. TURNER, *Biochem. J.* **115,** 481 (1969).
[13] G. J. KELLY and J. F. TURNER, *Biochim. Biophys. Acta* **208,** 360 (1970).

from the most immature tissue shows the greatest regulatory control and that from mature and senescent leaves shows the least.[7] Black and Wedding reported that PFK from dormant Jerusalem artichoke tuber discs was not inhibited by ATP when the discs had been 'aged' by washing,[8] and they attempted to explain the increase in respiratory rate during ageing of discs by the change in the sensitivity of PFK to ATP.

The activity of PFK from the slices at various culture stages was inhibited by ATP, citrate and PEP. The plots of PFK reaction velocity against F6P concentration gave a sigmoidal curve[10] and the apparent K_m of the enzyme for F6P was about the same for every PFK preparation examined. These results indicate that every PFK preparation possesses the same allosteric properties and that the change in glycolytic activity during culture is not caused by the change in the sensitivity of PFK to the effectors.

As cited above, Black and Wedding reported that 'ageing' changes the sensitivity of PFK to ATP in Jerusalem artichoke tubers. No such effect of 'ageing' was seen with carrot root slices, although slices were aged by the same method as theirs. In order to know whether or not this disagreement of our results with Black and Wedding's is due to the difference in materials, the properties of PFK from 'aged' slices of dormant Jerusalem artichoke tubers were investigated. Contrary to their results, our PFK preparations from 7 hr aged slices which exhibited maximal respiratory rate, and from 18 hr aged ones (they used 18 hr aged slices as the experimental material) showed almost the same allosteric properties [Figs. 2, 3(a) and (b)].

The difference between our results and those of Black and Wedding may be due to the fact that the latter do not use Polyclar AT in their extraction. But the effect of ATP on the activity of the PFK preparations which were extracted from 18-hr aged discs of Jerusalem artichoke tuber with or without Polyclar AT was similar. Therefore, it does not seem that the loss of the regulatory properties of PFK reported by them may be caused by the presence of phenolic substances which would exist in larger amounts when Polyclar AT was not included in the extraction medium. The discrepancy, thus, seems to be due to some other differences in technical procedure or in the physiological stage of materials used.

From the results described above the increase in respiratory rate at an early stage of culture of carrot root or during ageing of carrot root and Jerusalem artichoke tuber slices seems to be due not to the change in regulatory properties of PFK but to other mechanism such as the change in the amounts of some effectors of PFK, the *de novo* synthesis of respiratory enzymes or the rise of ATP turnover rate.

EXPERIMENTAL

Plant materials. Mature roots of a carrot cultivar, *Daucus carota* 'Oogata-Sanzun' and Jerusalem artichoke (*Helianthus tuberosus*) tubers harvested in December 1970 and stored in damp sand in a cold room at 0–4° for 3 months were used.

Methods of culture. Carrot-root phloem slices, 0·3 × 15 mm dia., were cultured as described in a previous paper.[9] The culture medium was composed of Murashige and Skoog's basal nutrients,[14] agar (0·6%) and 2,4-dichlorophenoxyacetic acid (5×10^{-6} M). The pH of the medium was adjusted to 5·7 with KOH before autoclaving. The cultures were maintained in the dark at 27°.

Measurement of respiratory gas exchange. Gas exchange was measured by standard Warburg manometric techniques. O_2 uptake and CO_2 output rates were determined at 30° over a 2 hr period after temperature equilibration.

Preparation of PFK. Samples (*ca.* 100 g fr. wt) were homogenized in a Waring blendor with 200 ml of ice cold 50 mM imidazole-HCl buffer (pH 7·8) containing 50 mM 2-mercaptoethanol, 2 mM EDTA and 10%

[14] T. MURASHIGE and F. SKOOG, *Physiol. Plantarum* **15**, 473 (1962).

activated Polyclar AT. The homogenate was filtered through cheese cloth and the filtrate was centrifuged at 70 000 *g* for 30 min at 2°. The supernatant was adjusted to pH 7·8 with NH_4OH and the protein fraction precipitating between 35 and 45% $(NH_4)_2SO_4$ saturation was collected according to Dennis and Coultate.[10] This precipitate was taken up in 3–4 ml of 50 mM imidazole–HCl buffer (pH 7·0). In the case of Jerusalem artichoke PFK, the enzyme fraction precipitating between 30 and 45% $(NH_4)_2SO_4$ saturation was collected according to Black and Wedding.[8] The activities of NADH oxidase and ATPase contained in the preparation were checked and PFK activity was corrected, the correction amounting to at most 4% of absorbance. Polyclar AT (insoluble polyvinylpyrrolidone) was activated by the method of Andersen and Sowers.[15]

Assay of PFK activity. PFK activity was determined by a modification of Uyeda and Racker's method.[16] The reaction mixture for the standard assay contained 50 mM imidazole–HCl buffer (pH 7·0), 100 mM 2-mercaptoethanol, 4 mM $MgCl_2$, 1 mM F6P, 1 mM ATP, 0·1 mM NADH, 0·5 mg (*ca.* 4·5 units) aldolase, 0·1 mg (*ca.* 4 units) glycerophosphate dehydrogenase, 0·01 mg (*ca.* 12 units) triose phosphate isomerase and 0·1 ml of the enzyme preparation. The reactions were started by addition of the enzyme preparation. After mixing, absorbance at 340 nm was read at 15 sec intervals for 5–10 min at 25°.

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